Preparation of Collagen–Polyurethane Composite Film and Its Subcutaneous Implantation in Rats: The Improvement of Tissue Compatibility

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ABSTRACT: This paper reports a safe, easy, effective, and one-step process to introduce a collagen layer onto a polyester-urethane surface for improving its biocompatibility and reducing acute inflammatory reaction. Collagen gel (COL) was spread onto the plasma-treated polyurethanes (PU) film to make PU–COL composite film by lyophilization. In this process, collagen on the interface was covalently immobilized to PU surface. Density of immobilized collagen molecules was examined to find the optimal experiment condition. The surface properties of the immobilized film were characterized by attenuated total reflection infrared spectrum and X-ray photoelectron spectroscopy to determine the efficiency of collagen immobilization. The results indicated that collagen chains had been grafted on PU surface because of plasma activation. To see if collagen modification can deduce acute inflammatory reaction and improve tissue guide regeneration, PU and PU–COL composite film were implanted subdermally in rats to analyze the effect of collagen immobilization. The reaction interface of PU–COL composite film and rat's tissue was observed by transmission electron microscope to analyze biocompatibility of PU–COL; unmodified PU film was used as control. The result showed that acute inflammatory reaction induced by PU–COL composite film had vanished gradually after 7 days and the material was embedded by tissue, almost forming a capsule. The capsule's wall thinned out gradually in the following days. Although the control group's inflammatory reaction did not vanish in 1 month and PU film embed in rat's tissue incompletely, PU implant migrated easily from the implant site. As a result, PU–COL composite film had most advantage in tissue guide regeneration and compatibility. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 1832–1841, 2006

Key words: polyurethane; collagen; plasma; surface; biocompatibility

INTRODUCTION

Polyurethanes (PU) are popularly used in implantable medical devices because of their good biocompatibility as well as mechanical properties.[1] However, the applications of PU in certain implant devices and particularly long-term implants have been limited because of their relative instability in the bioenvironment.[2] When acute inflammatory response is induced by PU implant, monocyte-derived macrophages that release reactive oxygen species are activated. They are the most abundant cells at the surface of PU implanted devices.[3] For long-term implantation, PU implants exhibit several problems. Polyester-urethane can be susceptible to degradation[4–5] and polyether-urethane to oxidation[6] gradually by hydrolytic enzymes and oxygen radicals, such as papain,[7,8] and enzymes derived by inflammatory cell (especially monocyte-derived macrophages), such as lysosomal enzymes,[9] cholesterol esterase, elastase, and carboxyl esterase[10,11].

As reported, good biocompatiblility of implant materials can lead to lower level of hydrolytic enzymes, which are activated by acute inflammatory reaction; that is, reduction of acute inflammatory reaction is an effective way to avoid degradation and oxidation of PU implants in harsh environment of living body. To decrease inflammation, enchancing biocompatibility of PU implant is an efficient method. And PU stability upon implant will be improved.[9,12]

Surface modification is a widely adopted method to improve biocompatibility of biomaterials such as PU.[13–16] Type I collagen as a robust fiber protein and main component of the extracellular matrix of most tissues is increasingly immobilized on different substrates for surface modification of biomaterials so as to improve biocompatibility.[17–19] Jong-Chul Park et al. treated PU surface with ozone and then immobilized collagen on PU substrate to improve cell attachment, proliferation, and collagen synthesis. The result indicated that the PU surface was effectively covered with type I atelocollagen. Attachment

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TABLE I
Standard Concentration of Collagen and Corresponding Absorbance

Concentration (μg/mL)	Absorbance (A_{595})
0	0
1	0.002
2	0.005
3	0.014
4	0.020
5	0.023

and proliferation of fibroblasts on the grafted type I atelocollagen were significantly enhanced, and it was assumed that the atelocollagen matrix supported the initial attachment and growth of cells.[20] van Wachem used collagen-immobilization on PU surface to enhance the latter's biocompatibility. PU surface was first treated by acrylic acid (AAc). Then collagen was covalently coupled onto PU surface by reaction with AAc. The results showed that collagen-immobilization of PU can increase the tissue integration.[21] Lee et al. immobilized collagen on PU surface through 1,2-bis(2,3-epoxypropoxy) ethane linking to improve the endothelial growth on PU substrate. In conclusion, PU surface modification with collagen can support the growth of endothelial cells. Such material may have good potential as vascular bioprostheses.[17]

All the methods they used were to introduce carboxyl and peroxide groups on PU surface to immobilize collagen molecules. The functional groups can also be introduced by low-temperature plasma technique. The technique not only has the same ability to introduce these groups on PU surface but also has unique advantages including conformative ultrathin-film deposition, good adhesion to substrate materials, and stable, durable characters without changing the bulk properties of the substrates.[22–25]

In this study, collagen reacted with carboxyl and peroxide groups on the plasma-treated PU substates. The one-step process reported in this paper to prepare polyurethanes–collagen gel (PU–COL) composite has several advantages compared with other methods. First, this method simplifies the preparation process with effective grafting efficiency. Second, this process avoids the risk caused by residual low-molecular weight regent such as acrylic acid, which may lead to inflammatory reaction. Third, low temperture plasma treatment can lead little damage to the bulk properties of PU substrate. Polyester-urethane was used in this study. To avoid PU biodegradation by hydrolytic enzymes produced by acute inflammatory reaction, surface of PU film was treated by low temperature plasma, then collagen was spread on PU, and lyophilized to make composite film.

EXPERIMENTAL

Collagen preparation

Collagen gel was extracted from bovine tendon in our laboratory[26] as follows: bovine tendon was cut into thin pieces (about 1 mm), and then dispersed in 0.3% acetic acid and ficin (activity 1 : 50,000; Sigma Chemical Co., St. Louis, MO) solution. The mixture was stirred intermittently for 3 days and then centrifuged by high speed freezing centrifuge. The supernatant was retained as the coarse collagen gel and purified by salting out and dialysis to get high purity collagen gel.

Preparation of COL–PU composite film

The PU film was polyester-urethane (type: SCU-PU-25), made in the department of polymer science and materials at SiChuan University. Number-average molecular weight of PU was 4×10^4– 6×10^4. In the preparation of SCU-PU-25, additive polymerization reaction was carried out with isocyanate and terminal hydroxyl polyester, using 1,4-butanediol as an extender.

High frequency low temperature plasma generator type GP300−6 (Electronic Machine Factory of Nong An Broadcast Television Bureau, JiLin Province, China) was used to treat PU films that were cut into quadrate shape (20×20 mm^2) in oxygen atmosphere. The experiment powers were set at 50, 60, 80, and 100 W and the treatment times were 2, 5, 8, and 10 min, respectively.

PU film was cut into square shape and both surfaces of PU film were treated with oxygen plasma with the optimal parameters. Length a (mm) and breadth b (mm) of PU film were measured by vernier caliper. Purified collagen gel, with an adjusted viscosity of 0.3% (w/v) propandioic acid was added dropwise on the plasma-treated PU film's surface (P-PU) until the

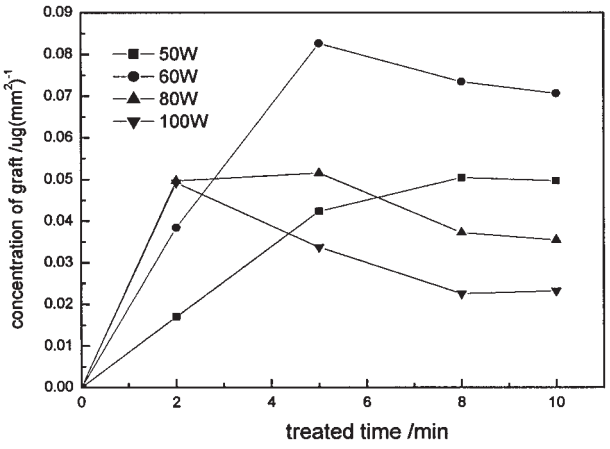

Figure 1 Density of collagen immobilized on PU surface.

whole surface was coated with collagen gel on both surfaces. The sample was refrigerated at 4°C for 12 h for collagen immobilization and then frozen at −30°C for 24 h. PU–COL composite film was obtained by lyophilization (Beijing boyikang Lab Instrument Co., Ltd., China).

Graft density

After collagen immobilization experiment, the sample was taken out from refrigeration, rinsed with 0.3% (w/v) propandioic acid 4–5 times, and then with deionized water 5–6 times to wash away unreacted collagen molecules. The sample was then dried in air. The dry PU film was put into 4% (w/v) propandioic acid solution (4 mL) and shook to uniformity, and allowed to stand at 4°C for 1 h. To 1 mL mixture, 0.15 mol/L sodium chloride solution (1 mL) and Coomassie brilliant blue solution (5 mL) was added, mixed uniformly, and allowed to stand for 10 min. Colorimetric method was used to measure absorbance of the solution at 595 nm (A_{595}), and then density of grafted collagen ([M], $\mu g/mm^2$) was calculated by eq. (1):

$$[M] = \frac{A_{595}}{a \times b \times 0.00467} \times 2 \qquad (1)$$

Standard curve was obtained as follows: Coomassie brilliant blue (Sigma, St. Louis, MO) solution including 0.01% (w/v) Coomassie brilliant blue, 4.7% (w/v) ethanol, and 8.5% phosphtic acid, was prepared to dye collagen. Standard curve equation of collagen concentration was calculated as follows: Collagen concentration was measured by the method of biuret that used concentration of bovine serum albumin as control. A series of collagen solutions were dyed by Coomassie brilliant blue solution, and the concentrations were 1, 2, 3, 4, and 5 μg/mL, respectively. Type 721 spectrophotometer was used to measure absorbance of collagen solution at the point of 595 nm, and then the standard curve equation of collagen concentration was calculated.

Surface characterization

X-ray photoelectron spectroscopy (XPS) was obtained by type VG ESCALAB Mk II spectrometer with a Mg Ka X-ray source (1253.6 eV photons) at a constant retard ratio of 40. The power of analysis was 216 W and the take-off angle was fixed at 45°. A survey scan spectrum was taken and surface elemental stoichiometries were determined from peak-area ratios. The high-resolution C_{1s} were curve-fit by a built-in software to analyze the chemical bonding states of atoms.

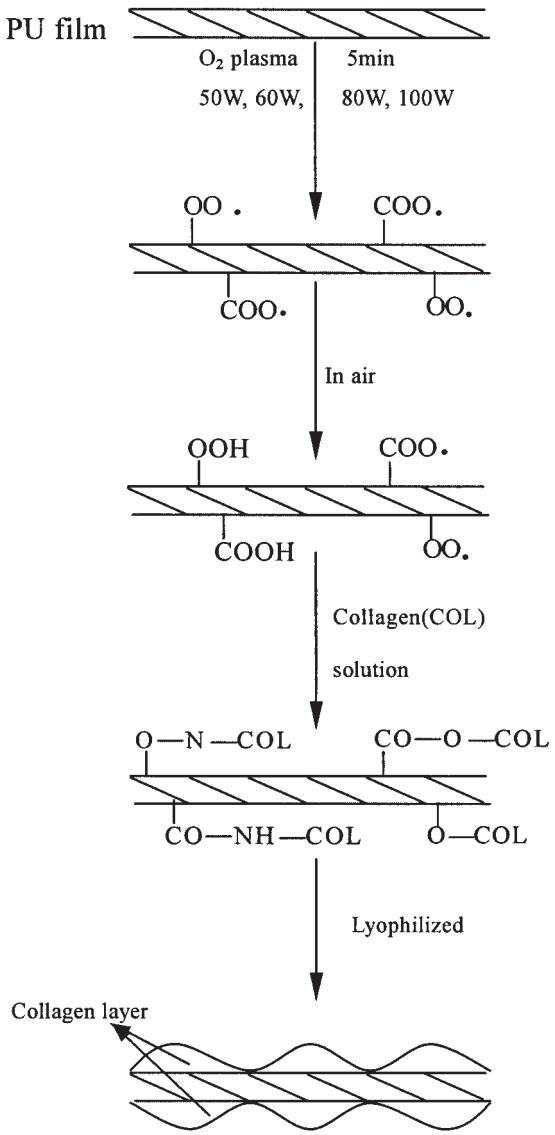

Figure 2 The mimic steps of immobilized collagen on PU substrate and the making of the composite film.

The attenuated total reflection infrared (ATR-IR) spectrum of PU, P-PU, and COL–PU films were obtained from a Nicolet nexus 670 spectrophotometer (Nicolet, USA), and a total of 32 scans were accumulated at a resolution of 4 wavenumbers.

Implant experiment

PU and PU–COL composite films were cut into 1 × 1 cm^2 and sterilized by Co_{60}-γ ray with radiation dose 25 kGy. Then they were implanted subdermally in ten healthy wistar rats with average weight about 200 g. The rats were numbered in two groups, such as group 1 for PU–COL composite implant and group 2 for PU implant. The rats were killed at days 2, 5, 15, 20 and 30

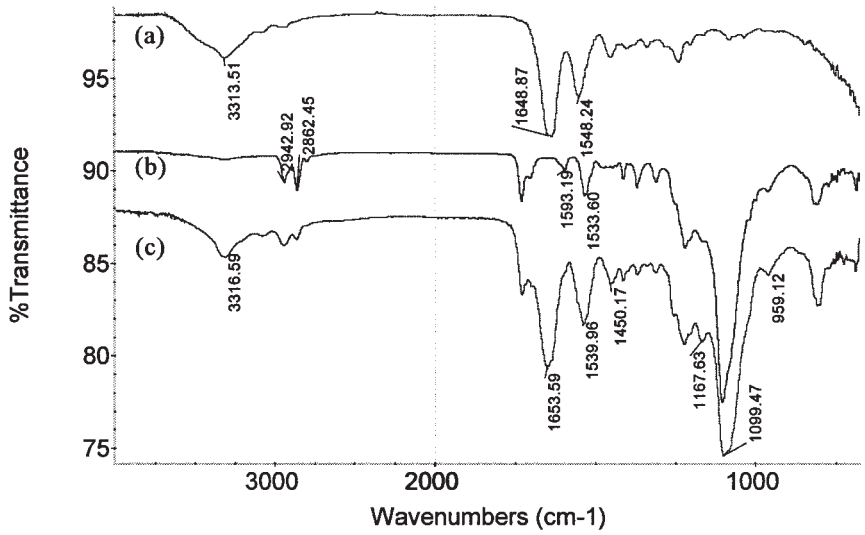

Figure 3 ATR-FTIR spectra of (a) PU, (b) PU-Cal, and (c) collagen film.

after implantation to observe the reaction of the body tissue, and the samples with the surrounding tissue were taken out. The implant experiment was carried out at the Center of Animal Experiment, Second Hospital of Harbin Medical University.

Transmission electron microscope (TEM)

The sample tissue (with implanted material in it) was first fixed with 2.5% (w/v) glutaraldehyde and then with osmium tetroxide. Then it was dehydrated by

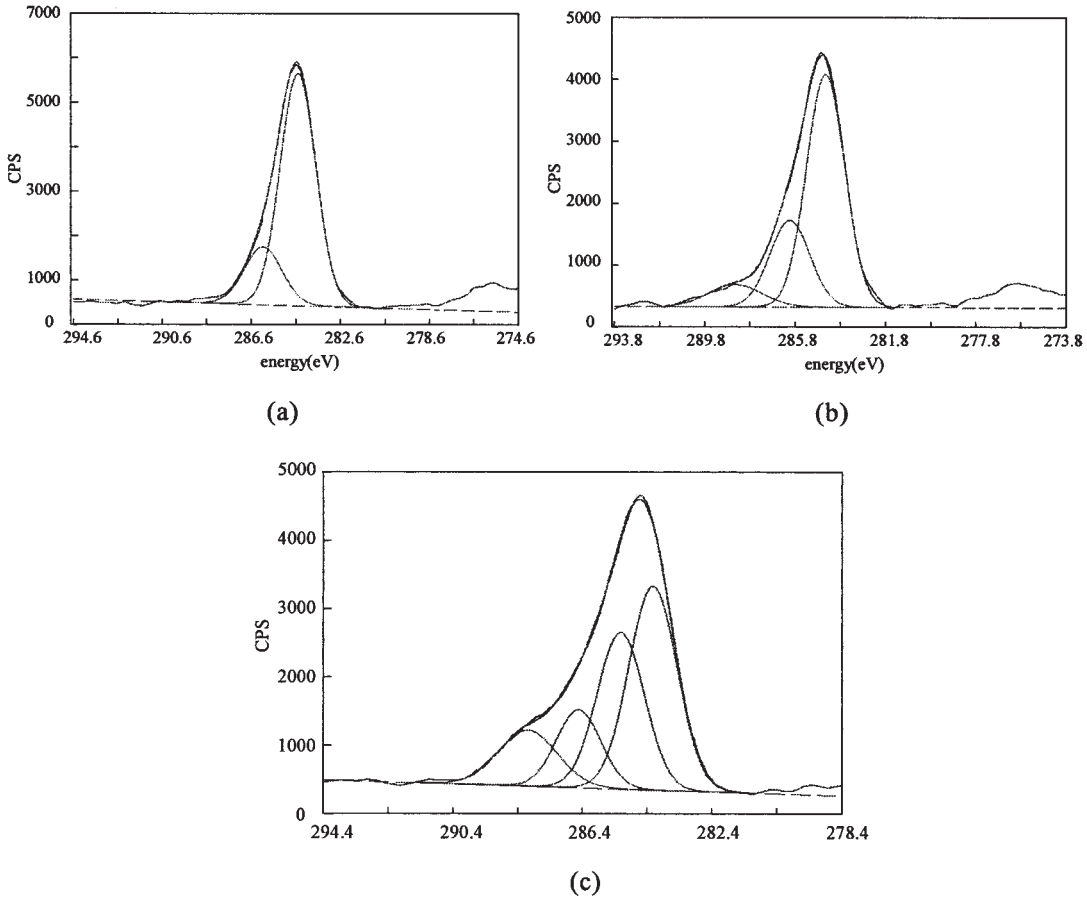

Figure 4 XPS C_{1s} divide peak chart: (a) PU film's C_{1s}; (b) P-PU film's C_{1s}; (c) PU–COL film's C_{1s}.

TABLE II
Fraction of Carbon Functional Groups from High-Resolution C_{1s} XPS Peaks

	Content of functional groups (mol %)			
Sample	C—H (284.2 eV)	C—O—C*, C—N (285.2 eV)	C—OH (286.5 eV)	O—C═O (288.4 eV)
PU	79.16	—	20.84	—
P-PU	65.60	—	25.59	8.81
PU-COL	40.94	30.83	14.53	13.70

50% (w/v) acetone for 10 min, 70% (w/v) for 10 min, 90% (w/v) for 10 min, and 100% (w/v) acetone for 15 min, and embedded in epoxy resin (Super) that was cured at 70°C for 8 h. Ultrathin section of the tissue (70 nm) was cut by ultramicrotome to observe interface of implant material and body tissue with TEM (JEOL, JEM-1200EX, Tokyo, Japan) to evaluate biocompatibility.

RESULTS AND DISCUSSION

Preparation of PU–COL composite film

Standard curve equation for concentration of collagen solution (C, μg/mL) was obtained by measuring absorbance of collagen solution in 595 nm before calculating density of grafted collagen on PU substrate. Relationship between concentration of collagen and absorbance in 595 nm is listed in Table I, relational equation [eq. (2)] was obtained by linear regression calculation, and the regression coefficient (R^2) was 99.95%.

$$A_{595} = 0.00467C \quad (2)$$

The density of grafted collagen is shown in Figure 1. Under 50 W plasma treatment, the density of grafted collagen was first increased greatly and then mildly with the increase of treated time. The highest point appeared when the power for plasma was 60 W (Fig. 1). The optimal experiment parameters were corresponding to this point, which was treatment at 60 W for 5 min. When PU film was treated with higher power (80 W, 100 W), density of collagen on PU surface was increased first and then decreased, but the inflection value was lower than that for 60 W. That is to say, higher power for plasma treatment was not suitable for producing more active point for collagen molecules grafted on PU surface.

The mimic steps of collagen immobilized on PU surface were shown in Figure 2. Active C—H groups on PU surface were easy to be dehydrogenized and oxidized by oxygen plasma into carbonyl groups, carboxy groups, peroxide groups, and free radical groups. These groups reacted with amino residue and carboxy groups on collagen chains, so that collagen can be immobilized on PU surface by covalent bond.

When power and time of plasma treatment were increased, active particles of oxygen plasma and carbonyl groups, carboxy groups, peroxide groups, and radical groups on PU surface were increased. More collagen molecules can be introduced. Then concentration of collagen on PU surface was increased with increase in the plasma treatment time and power at first stage. Simultaneously, sculpture and oxidation caused by oxygen plasma were raised. When treatment time and power were increased to a given number (such as 5 min, 60 W), radical groups on PU surface were annihilated quickly and degree of oxidation was enhanced at the same time. As a result, the number of carbonyl groups was higher than that of carboxy groups. So, when treatment time and power reached a critical point, the amount of caboxyl groups

TABLE III
Macroscopic Observation of Tissue Reaction to PU and PU-COL Composite Film Implantation

Days of implant	Group 1: PU-COL composite film	Group 2: PU film
2	Inflammatory reaction: conglutination reaction, accretion, local tumefaction, harden	Inflammatory reaction: accretion, local tumefaction, harden
7	Exist hard bulk, becoming capsule; inflammatory was attenuated	Exist hard bulk clearly, not encapsuled by tissue; inflammatory reaction: conglutination
15	Encapsuled completely; inflammatory vanished	Not encapsuled by tissue; inflammatory reaction: conglutination
20	Capsule become attenuated	Partly fraction encapsuled, partly by tissue; inflammatory reaction: conglutination
30	Capsule become attenuated continuously	Partly encapsuled by tissue

and radical groups arrived at a maximum value and then decreased with the increase in treatment time and power. This contributed to the initial increase in immobilized collagen with the treatment of power and time, which was followed by a decrease. The sample treated with power of 60 W for 5 min was selected as the optimal experiments parameter for the following work.

ATR Fourier transform IR (ATR-FTIR)

The surface structure of the modified film was studied by ATR-FTIR. Figure 3 showed the FTIR spectrum of the pristine collagen film (spectrum a), PU film (spectrum b), and PU–COL film (spectrum c), respectively. In spectrum (c), the major absorption peaks appeared at 3316.59, 1645.02, 1552.17, and 1099.47 cm^{-1}. The same peaks were also found in spectrum (a) and (b). After immobilization, some signals on spectrum (b) were weakened for peak 1099.47 cm^{-1} (from C—O stretching vibrations), peak 2942.92 cm^{-1} and peak 2862.45 cm^{-1} (from CH_2 framework stretching vibrations). In spectrum (c), peak 1593.19 cm^{-1} raised from C—O—C stretching vibrations was vanished. And peak 1533.60 cm^{-1} was widened, peak 3316.59 cm^{-1}, peak 1645.02 cm^{-1}, and peak 1450.17 cm^{-1} appeared in spectrum(c), which were the characteristic peaks of collagen. Generally, amide A bands (3315.51 cm^{-1}) arised from N—H stretching vibrations absorption, amide I bands (1645 cm^{-1}) originated from C═O stretching vibrations coupled to N—H bending vibration, and the amide II bands(1552.17 cm^{-1}) arised from the N—H bending vibrations coupled to C—N stretching vibrations in spectrum (a).

Spectrum (c) showed that the collagen had been successfully immobilized on the PU surface. From the analysis of ATR-IR spectrum, it was indicated that when PU film was treated with oxygen plasma, C—O—C bonds on the surface of PU film was activated first to form radical groups. These radical groups reacted with collagen molecules or other radical groups in air to form carboxy groups and peroxide groups, and then collagen reacted with these groups and immobilized on the PU surface.

XPS

The high-resolution C_{1s} were curve-fit in Figure 4. In the case of PU film, the C_{1s} core level spectrum [as seen in Fig. 4(a)] contains two major peak components, with binding energy at 284.6 eV for the C—H species and at 286.2 eV for the C—O species. After PU was treated with oxygen plasma [as seen in Fig. 4(b)], another peak component appeared with binding energy at 288.4 eV for the O—C═O species. After collagen immobilization onto the film surface [as seen in Fig. 4(c)], the C_{1s} core level can be curve-fitted with four peak components, with binding energy at 284.4 eV for the C—H species, at 286.5 eV for the C—O species, at 288.4 eV for the O—C═O species, and at 285.2 eV for the C—N and C—O—C^* species.[13,22,27,28] The comparative contents of each species were listed in Table II.

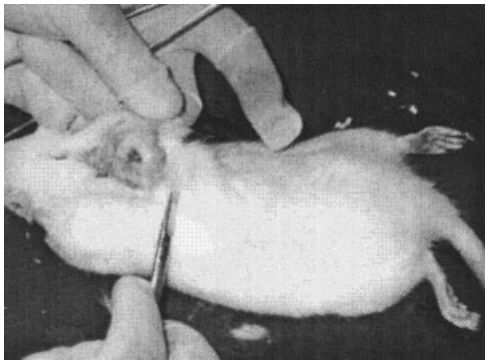

Figure 5 Appearance of capsule in 15 days

Compared with Figure 4(a,b) and Table II showed that there was a new peak in 288.4 eV that was assigned to O—C═O come into being after oxygen plasma treatment, and comparative content of O—C═O was 8.81%. An increase in 286.5 eV was assigned to C—OH in Figure 4(b), which indicated the content of C—OH groups that had been raised to 25.59%. These groups provided the advantage for collagen to graft on PU surface. These changes were attributed to the oxidation induced by plasma treatment. While a new peak in 285.2 eV assigned to C—N species and C—O—C^* species was emerged in Figure 4(c) and content of the groups was high to 30.83%, the content of C—OH species had been reduced to 14.53%. The results indicated that the surface properties of PU subsrate were changed by collagen modification. C—N species were amide residues and peptide bonds on collagen chains, not the amido bonds in PU surface, because amido bonds were in hard segment of PU. When PU was come into being, hard segment was spreaded in soft segment (polyether) and under the surface of PU. The emergence of C—N proved that collagen has been immobilized on PU surface. The reduction of C—O species and the emergence of the C—O—C^* species indicated that the oxide or peroxide groups had been decomposed and collagen chains grafted with C—O species by covalent on PU surface.

The results of ATR-IR and XPS demonstrated that oxygen plasma treatment was an effective way to activate the functional groups on PU surface to immobilize collagen on PU substrate and provided evidence of the mimic steps of collagen immobilization.

Macroscopic observation of implant

Table III shows the macroscopic observation of tissue reaction to PU and PU–COL composite film implan-

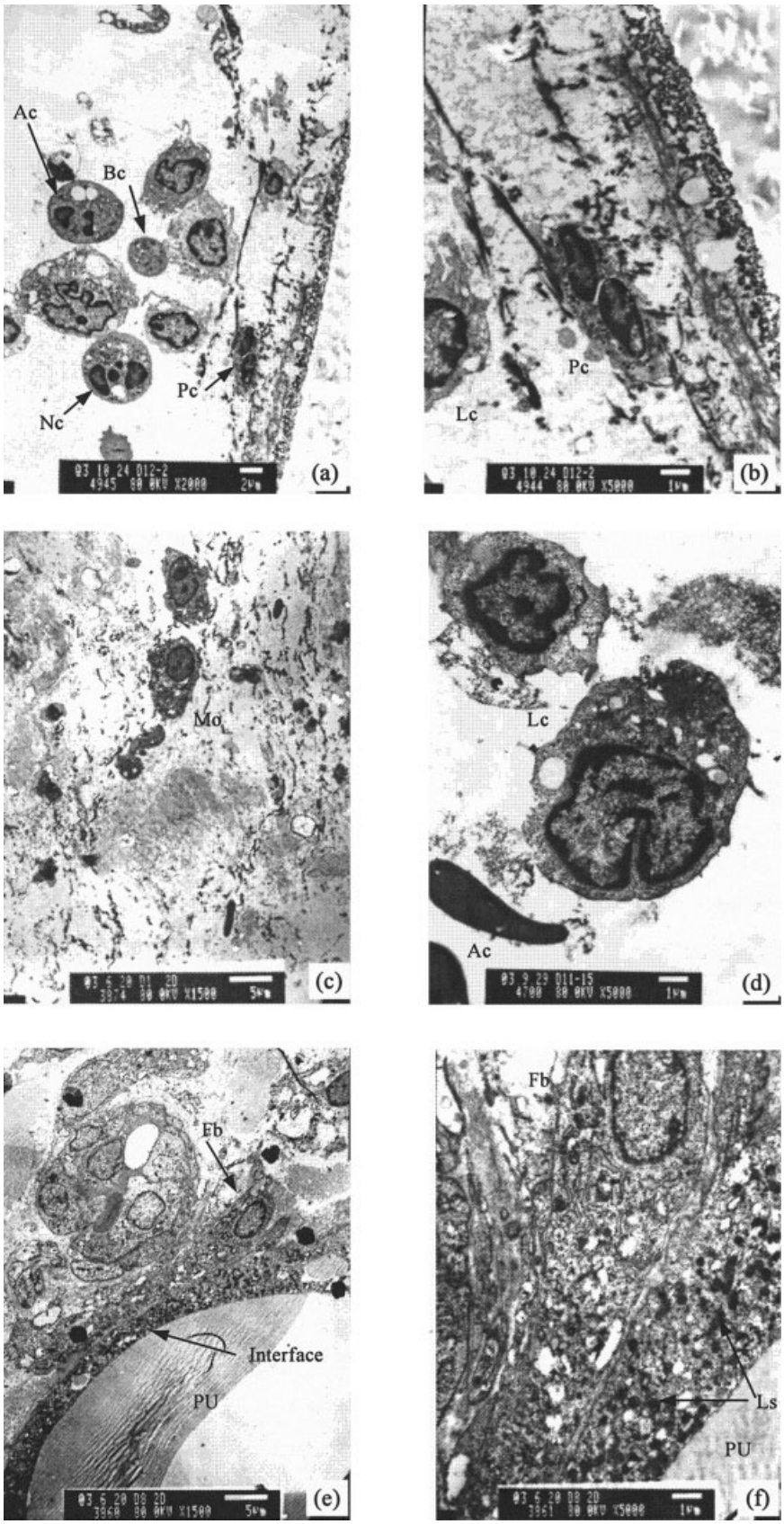

Figure 6 TEM images of (a–d) PU and (e–h) PU–COL implant at day 2: (a) leukocyte (including acidocyte, neutrophilic granulocyte, basophilic granulocyte, and pathologic cell) around PU film (×2000); (b) pathologic cell fractionated gain (×5000); (c) macrophage in surrounding tissue of PU (×1500); (d) leukocyte (monocyte) and akaryocyte beside PU (×5000); (e) interface of tissue and PU–COL composite film (×1500); (f) fractionated gain of fibroblast in (e) and interface (×5000); (g) some fibroblasts around PU–COL composite film and some monocytes beside composite film (×2000); (h) collagen on the surface of PU and dispersed in tissue (×10000).

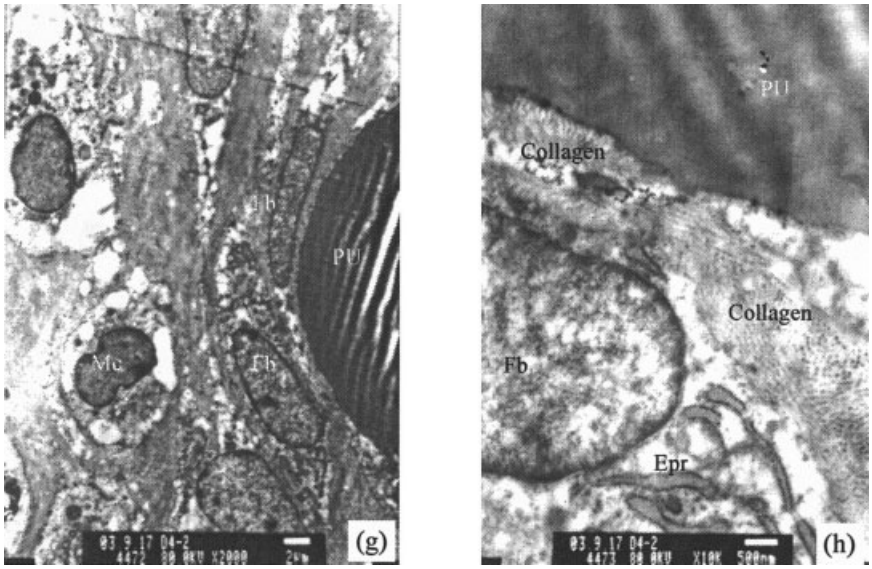

Figure 6 (*Continued from the previous page***)**

tation during 30 days. At first two days, both PU film and PU–COL composite film induced inflammatory reaction that included accretion, local tumefaction, and local tissue becoming hard. In group 1, inflammatory induced by PU–COL composite film implant was attenuated after 7 days, and capsule came into being, and wall of capsule was thinned out gradually in the following days. The inflammatory reaction lasted for more than 20 days after PU film implant in group 2; at the same time, PU film was not encapsuled completely by tissue up to 30 days after implantation. The results indicated that tissue regeneration and compatibility of PU–COL composite film was better than that of PU film, that is, PU–COL composite film implant can reduce acute inflammatory reaction and emergence of macrophage that was induced by inflammation. Figure 5 shows the appearance of capsule for PU–COL composite film implant in 15 days.

Observation of implant by TEM

Figure 6 shows the TEM image of implants of PU and PU–COL composite film in the second day. PU as foreign substance implanted subcutaneously was attacked by leukocytes (Lc), which include acidocyte (Ac), neutrophilic granulocyte (Nc), basophilic granulocyte (Bc) and macrophage (Mo), in the second day [Figs. 6(a), 6(c), and 6(d)]. These cells were induced by acute inflammatory reaction. Interface between PU and body tissue [shown in Fig. 6(b)] was disheveled phospholipid layer, cell fragments, and sap, there was a pathologic cell induced by PU implant in the image, color of the cell nucleus became dark. The analysis indicated that when PU was implanted subdermally in rats, acute inflammatory reaction occurred and PU was exposed to leukocyte directly. With pathologic cells' death and continuous inflammatory reaction, property of PU implants would be influenced by enzyme and cytokine produced by inflammatory cells such as Mo.

While when PU–COL composite film was implanted subcutaneously in rats, light acute inflammation occurred and no inflammatory cells were observed on the surface of PU of composite film in TEM images as showed in Figures 6(e) and 6(g). Figure 6(e) shows that between PU and tissue there was a layer of transition (a layer of collagen), an outer transition layer (fibroblasts (Fb)), and tissue cells that were adhered on the collagen layer. Many lysosomes existed in transition layer as showed in Figure 6(f). And collagen would be degraded by these lysosomes in the following days of implantation and the existence of lysosome was a proof that the cell here was active. Figure 6(g) shows some fibroblasts that had been grown inside of collagen layer of PU–COL composite film and some monocytes (Mc) dissociated in tissue. It was observed that PU did not expose directly to inflammatory cells. There were many collagen adhered on the surface of PU [Fig. 6(h)], it was also a demonstration that collagen chain had been immobilized on PU surface. The results indicated that PU–COL composite film avoided acute inflammatory reaction, as PU substrate was protected by collagen layer.

Figure 7 shows the images of implants of PU and PU–COL composite film at day 20. There were a small quantity of fibroblasts and collagen near the surface of PU film [Fig. 7(a)]. Between the tissue and PU film, there was a blank zone that perhaps was tissue sag and the fibroblast did not adhere on PU surface [shown in Fig. 7(b)]. That is to say, PU implant was easy to move away from the implant site and hemopoietic tissue was easy to renegade from PU surface.

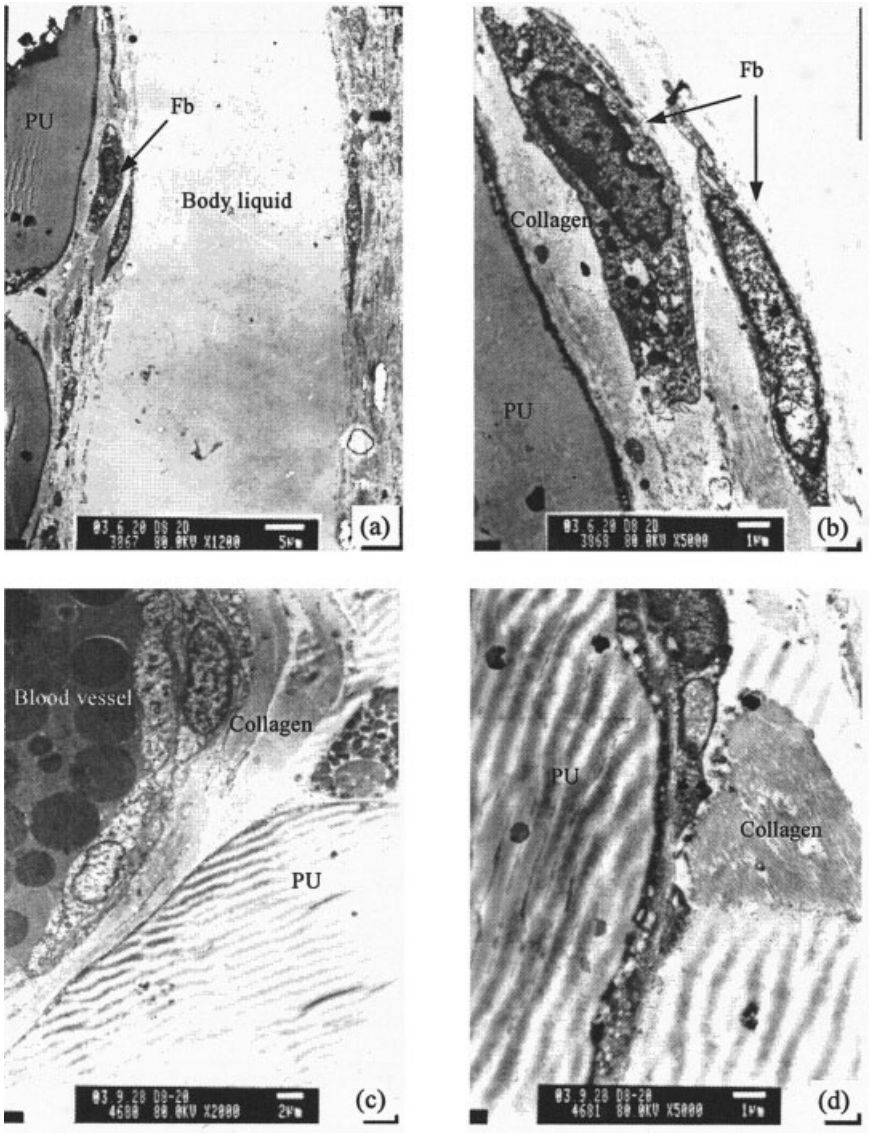

Figure 7 TEM images of (a,b) PU and (c,d) PU–COL implant at day 20: (a) interface of PU film and tissue (×1200); (b) fibroblast near PU surface (×5000); (c) blood vessel in capsule beside PU–COL composite film (×2000); (d) collagen on the surface of PU (×5000).

Tissues and cells were adhered on PU–COL composite film even at day 20, as shown in Figure 7(c). It was observed that a blood vessel was found in capsule beside PU–COL composite film and there were also many collagen fibers that were produced by fibroblast cell in tissue. These phenomena demonstrated that immobilized collagen had taken effect to improve tissue regeneration and biocompatibility. Cells adhered on the surface of PU were seen in Figure 7(d), and the interface of PU and tissue was phospholipid layer and tissue collagen.

CONCLUSIONS

A one-step process was used to immobilize collagen onto a PU substrate by spreading collagen onto oxygen plasma-treated PU surface. The optimal parameters of oxygen plasma treatment were obtained by Coomassie brilliant blue quantitative method, and the best conditions were that the power of plasma generator was 60 W and treatment time was 5 min. The results from ATR-IR and XPS indicated that collagen had been successfully immobilized on PU surface by covalent bonds. The optimal graft efficiency was about 0.0826 ug/mm^2. To evaluate the effect of collagen modification, PU and PU–COL composite films were implanted subcutaneously in rats to observe the inflammatory reaction by macrography and TEM upon 30 days of implantation. The results indicated that PU–COL composite film implant can reduce acute inflammatory reaction and that the inflammation induced emergence of macrophage would be dimin-

ished. Collagen layer can protect PU substrates *in vivo*. This PU–COL composite film by a one-step process is promising for biomedical application.

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